# Expression Profiling of Long Noncoding RNA in Plasma and Cell Lines Using the iScript™ Explore One-Step RT and PreAmp Kit

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## Abstract

Here we demonstrate a reverse transcription quantitative PCR (RT-qPCR) workflow, utilizing the iScript Explore One-Step RT and PreAmp Kit, that is applicable to the discovery and validation of long noncoding RNA (lncRNA) biomarkers. The workflow was used to analyze both lncRNA and mRNA in cell-free RNA (cfRNA) isolated from limited amounts of human plasma samples and was also applied to the detection of lncRNA in two common cell lines. By incorporating preamplification into the RT-qPCR workflow, we were able to detect 29 of 41 lncRNA and 8 of 15 mRNA targets in the cell-free RNA, as well as an average of 95 of 107 lncRNAs in the cell lines. The iScript Explore workflow was also compared to a traditional RT-qPCR workflow where it was found to improve detection of lncRNA, with targets detected an average of 10.1 cycles earlier.

## Introduction

Long noncoding RNAs form a large and diverse class of RNA molecules defined by their size (>200 nucleotides) and limited protein coding potential. While many thousands of lncRNAs are known to exist, the functions are known for relatively few, with the vast majority requiring further exploration to understand their role within the cell and in the context of disease. As research into these molecules grows, it will hopefully lead to new insights into the development of disease and to the discovery of new biomarkers.

However, studying lncRNA can be challenging. Sensitivity is often a limiting factor because these molecules are typically expressed at levels much lower than mRNAs.

In this study, we demonstrate that the iScript Explore One-Step RT and PreAmp Kit improves RT-qPCR detection of lncRNA and mRNA in cell-free RNA from human plasma samples. This all-in-one kit produces preamplified cDNA directly from up to 10.5 µl of RNA sample by combining the reverse transcription and preamplification steps in a single reaction. The workflow was also applied to the detection of lncRNA in two common cell lines.

## Materials and Methods

### Plasma RNA

Plasma samples from patients with prostate cancer and from healthy donors were obtained from Dx Biosamples. The Quick-cfRNA Serum & Plasma Kit (Zymo Research) was used to isolate cfRNA from 450 µl of plasma. The concentration of cfRNA was estimated using the Experion™ RNA High Sens Analysis Kit (Bio-Rad Laboratories, Inc.) and ranged from 170 to 910 pg/µl. The iScript Explore One-Step RT and PreAmp Kit (Bio-Rad) was used to eliminate genomic DNA and synthesize preamplified cDNA from 1,600 pg of cfRNA for each sample tested. Preamplification was performed for 14 cycles using pooled PrimePCR™ PreAmp Assays (Bio-Rad) for 41 lncRNA and 15 mRNA targets (Table 1).

To perform the qPCR, a 384-well custom PrimePCR Plate with predispensed primers was designed to analyze the lncRNA and mRNA assays along with PrimePCR Control Assays (Bio-Rad; 63 assays total). Preamplified cDNA was diluted tenfold with TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0) and 1 µl was used per 10 µl qPCR reaction, which also consisted of SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad) and nuclease-free water. Quantitative PCR was performed in triplicate on a CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad) with the PrimePCR cycling protocol (95°C for 5 min; 40 cycles: 95°C for 5 sec, then 60°C for 30 sec; melt curve from 65 to 95°C in 0.5°C increments).

The qPCR data were analyzed using CFX Maestro™ Software (Bio-Rad) and only reactions with a quantification cycle (Cq) value less than 30 were considered positive (this threshold is equivalent to using cycle 35 as a cutoff without preamplification). Using CFX Maestro Software, a Student

*t*-test (alpha = 0.05) was performed to compare the average target levels (relative quantities) between the cancer and healthy control groups.

#### Cell Line RNA

HeLa and K562 cells were grown to 80% confluence in 96-well cell culture plates (approximately $10^4$ cells/well). Cells were harvested by removing culture medium, washing once with PBS, and adding 50 µl of SingleShot™ Cell Lysis Master Mix prepared according to the manual for the SingleShot Cell Lysis Kit (Bio-Rad; catalog #1725080). Lysates were transferred to 96-well PCR plates and incubated at 37°C for 5 min, followed by 5 min at 75°C to eliminate genomic DNA.

Reverse transcription and preamplification were performed using the iScript Explore One-Step RT and PreAmp Kit along with pooled PrimePCR PreAmp Assays for 107 lncRNAs (Table 1). SingleShot lysate (2.5 µl) was used as input for each iScript Explore reaction. After 14 cycles of preamplification, each 50 µl iScript Explore reaction was diluted tenfold with TE buffer to a final volume of 500 µl. Diluted preamplified cDNA (2 µl) was used in each 10 µl qPCR reaction performed using SsoAdvanced™ Universal SYBR® Green Supermix and PrimePCR Assays for 107 lncRNAs that were candidates for the PrimePCR Pan-Cancer lncRNA Array (Bio-Rad).

Data were collected on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad), using the PrimePCR cycling protocol, and analyzed with CFX Maestro Software. Only results with a mean Cq less than 29 were evaluated (this threshold is one cycle earlier than the threshold that was used for cell-free RNA because twice as much preamplified cDNA was used as input for the qPCR).

#### Comparison to a Standard RT-qPCR Workflow

Universal human reference RNA (Agilent Technologies, Inc.) at 0.095 ng/µl was used to generate cDNA with and without preamplification using the maximum input sample volumes allowed by the respective workflows.

For the preamplification workflow, 10.5 µl (1 ng) of the reference RNA was used as the input for a 50 µl iScript Explore One-Step RT and PreAmp reaction with 26 PrimePCR lncRNA PreAmp Assays (Bio-Rad). The lncRNAs used were selected based on their expression level so that the targets could be readily detected in the non-preamplification workflow. After 14 cycles of preamplification, the resulting sample was diluted tenfold with TE buffer to a final volume of 500 µl.

For the non-preamplification workflow, 14 µl (1.33 ng) of the reference RNA was used as input for a 20 µl cDNA synthesis reaction using the iScript gDNA Clear cDNA Synthesis Kit (Bio-Rad). One microliter of the resulting cDNA samples from both workflows was used in a 10 µl qPCR reaction consisting of SsoAdvanced™ Universal SYBR® Green Supermix and PrimePCR lncRNA Assay (Bio-Rad).

Data were generated on a CFX384 Touch Real-Time PCR Detection System using the PrimePCR cycling protocol.

**Table 1. lncRNA and mRNA targets used for the analysis of cell-free RNA from plasma and cell line RNA lysates.**

| Plasma RNA Targets | | | Cell Line RNA Targets | | | | |
|---|---|---|---|---|---|---|---|
| *(BCDIN3D-AS1)* | *PCAT5* | *ERG* | *(AC109315.1)* | *KCNQ1OT1* | *lnc-FAM83B-1* | *lnc-SEPT7-3* | *RNF139-AS1* |
| *(CBR3-AS1)* | *(PCAT18)* | *FOLH1/PSMA* | *AP000221.1* | *LINC00493* | *lnc-FAM84B-3* | *lnc-SH3GL3-5* | *RP11-280G9.1* |
| *CDKN2B-AS1* | *(PCAT29)* | *GUSB* | *BANCR* | *LINC00662* | *lnc-FBXL2-2* | *lnc-SLC25A26-9* | *RP11-554I8.2* |
| *CFLAR-AS1* | *PCGEM1* | *HMBS* | *BCAR4* | *LINC00857* | *lnc-FNTB-1* | *lnc-SLC39A6-1* | *SCHLAP1* |
| *(CTBP1-AS)* | *PRNCR1* | *(KLK3)* | *CASC11* | *LINC00888* | *lnc-FRMD4B-3* | *lnc-TMEM14A-1* | *SNHG8* |
| *FGD5-AS1* | *PSMD6-AS2* | *PTEN* | *CBR3-AS1* | *LINC00957* | *lnc-GNG11-2* | *lnc-TRIM16-4* | *SNHG15* |
| *(GNG12-AS1)* | *PTENP1* | *REG3A* | *CCAT2* | *LINC01133* | *lnc-HIST1H2BF-1* | *(lnc-TSHZ2-1)* | *SOCS2-AS1* |
| *H19* | *(RNF139-AS1)* | *(SPDEF)* | *CCDC26* | *LINC01137* | *lnc-HYAL4-4* | *lnc-ZBTB17-1* | *SPRY4-IT1* |
| *(HAR1A)* | *(SCHLAP1)* | *TBP* | *CDKN2B-AS1* | *LINC01139* | *lnc-INTS12-1* | *lnc-ZNF460-2* | *SRA1* |
| *HAR1B* | *SNHG15* | *(TERT)* | *CTBP1-AS* | *(LINC01207)* | *lnc-JAG1-6* | *lnc-ZNF720-1* | *STARD4-AS1* |
| *HOTAIR* | *SNHG19* | *(TGM4)* | *DICER1-AS1* | *LINC01315* | *(lnc-KLHL31-8)* | *LUADT1* | *TINCR* |
| *KCNQ1OT1* | *SOCS2-AS1* | *(TMPRSS2)* | *DLEU1* | *LINC01419* | *lnc-LAMA2-3* | *MEG3* | *TP53TG1* |
| *LINC00339* | *(TINCR)* | | *DLX6-AS1* | *LINC01537* | *lnc-LRIT2-4* | *MNX1-AS1* | *TUG1* |
| *LINC00493* | *TP53TG1* | | *DSCAM-AS1* | *LINC-ROR* | *lnc-MARCH7-1* | *NADK2-AS1* | *(TUNAR)* |
| *LINC00662* | *(TSIX)* | | *EGOT* | *lnc-ACER2-1* | *lnc-MRPL14-2* | *NEAT1* | *UBA6-AS1* |
| *LINC01024* | *TUG1* | | *FGD5-AS1* | *lnc-ACOT13-1* | *lnc-PCDHGA1-2* | *NRSN2-AS1* | *WAC-AS1* |
| *LINC01116* | *UBA6-AS1* | | *FOXP4-AS1* | *(lnc-AF127577.1-2)* | *lnc-PKN3-1* | *PCA3* | *WSPAR* |
| *(MEG3)* | *WAC-AS1* | | *GAS6-AS1* | *lnc-ANKRD36C-9* | *(lnc-POTEG-5)* | *PCAT1* | *XIST* |
| *NEAT1* | *XIST* | | *GNG12-AS1* | *lnc-ARFIP2-2* | *lnc-PSMC5-3* | *PCAT29* | *ZFAS1* |
| *NORAD* | *AMACR* | | *H19* | *lnc-C17orf64-2* | *lnc-RNF24-2* | *PCGEM1* | |
| *PCA3* | *(AR)* | | *HIF1A-AS1* | *lnc-DDX18-1* | *lnc-RP11-180C1.1.1-2* | *PRNCR1* | |
| *PCAT1* | *CCND1* | | *HMGN3-AS1* | *lnc-DOCK8-4* | *lnc-SBDS-14* | *PTENP1* | |

Long noncoding RNA targets are shown in black and messenger RNA targets are shown in orange. Targets without parentheses were detected in at least one sample or cell line. Targets shown in parentheses were not detected in the respective experiments.

## Results

### Plasma RNA

Of the 41 lncRNA targets that were analyzed, 29 lncRNAs were detected in at least one sample and an average of 20 lncRNAs were detected in each sample (Figure 1). Of the 15 mRNAs that were analyzed, eight were detected in at least one sample and an average of eight were detected in each sample (data for mRNA not shown). Of the lncRNA and mRNA targets that were coincidentally detected in the prostate cancer and healthy donor groups, there were no significant differences in expression according to the Student *t*-test (alpha = 0.05). The ten lncRNAs that were detected in only one sample had Cq values close to the threshold at cycle 30, and no conclusions were drawn from these results. *HOTAIR* was detected in both the healthy controls but not in the patients with prostate cancer. However, the results for the healthy controls were also close to the threshold at cycle 30.

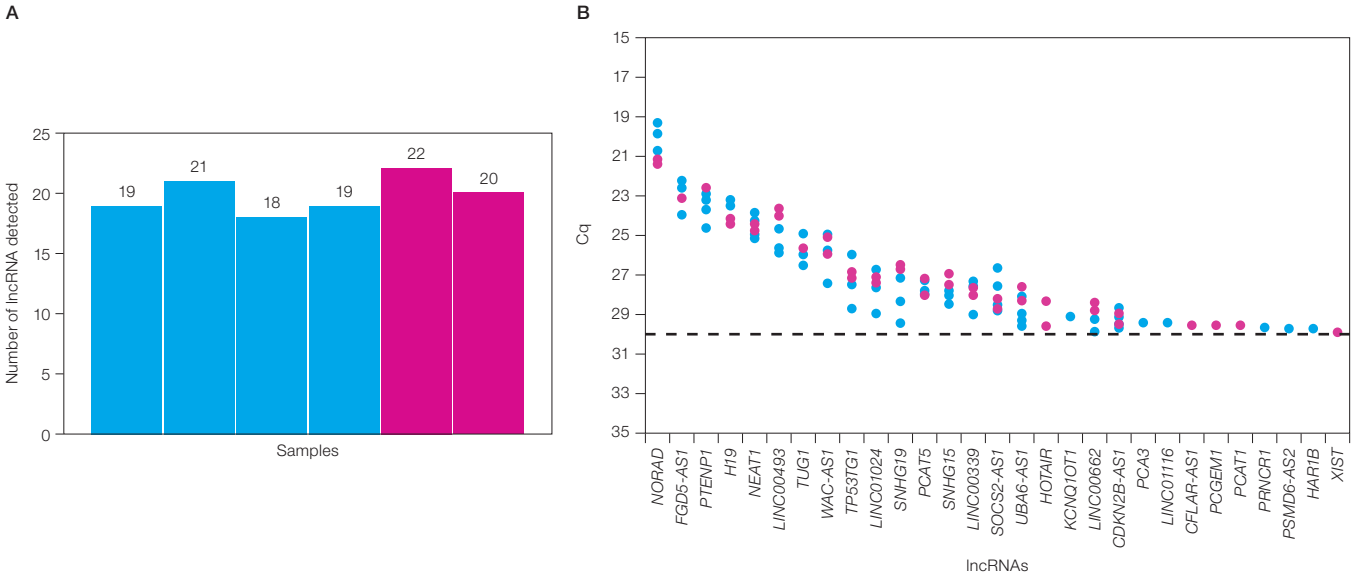


**Fig. 1. Analysis of lncRNA in cell-free RNA from plasma samples.** lncRNAs (41) were analyzed in cell-free RNA that was prepared from the plasma of four patients with prostate cancer and two healthy donors. RT-qPCR analysis using PrimePCR lncRNA Assays was performed on preamplified cDNA that was generated directly from the cell-free RNA samples using the iScript Explore One-Step RT and PreAmp Kit. **A**, between 18 and 22 lncRNAs were detected with a Cq <30 in each of the cancer (■) and healthy control (■) samples; **B**, plot shows the qPCR results (Cq) for the detected targets in the cancer (■) and healthy control (■) samples. Cq, quantification cycle.

### Cell Line RNA

The number of detected lncRNA was evaluated for each cell line using the Cq cutoff at cycle 29. Using this cutoff, 96 lncRNAs (90%) were detected in HeLa and 94 lncRNAs (88%) were detected in K562 cells (Figure 2), respectively. In HeLa cells, the lncRNAs analyzed had a median Cq of 18.9, whereas in K562 cells, the median Cq was 21.0.

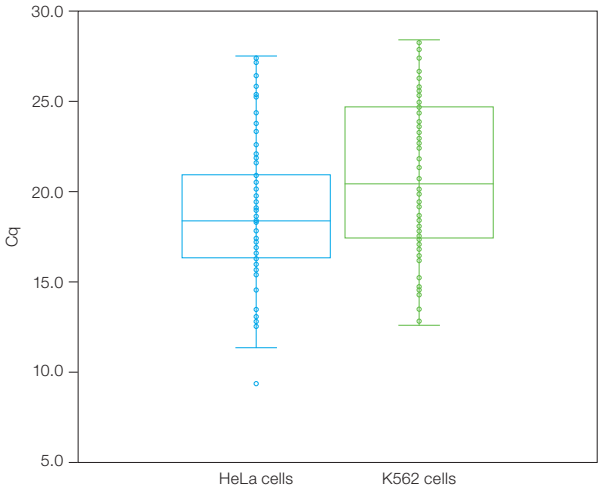


**Fig. 2. High rate of lncRNA detection in common cell lines.** lncRNA candidates (107) for the PrimePCR lncRNA Pan-Cancer Array were analyzed by qPCR in HeLa (■) and K562 (■) cell lines using preamplified cDNA prepared from cell lysates using a SingleShot and iScript Explore Kit–based workflow. The plot shows 96 (90%) and 94 (88%) targets detected in HeLa and K562 cell lines, respectively, using a Cq cutoff of 29 for positive results. HeLa cells had a median Cq of 19.4 with the 50% middlemost results in the range of 17.1 to 23.0. K562 cells had a median Cq of 22.2 with the middle 50% falling between 18.1 and 26.1. Cq, quantification cycle.

### Comparison to a Standard RT-qPCR Workflow

Nineteen lncRNAs were selected for comparing the iScript Explore workflow, which uses preamplification, with a non-preamplification workflow using the iScript gDNA Clear Kit. The Cq values from both workflows were compared (Figure 3). With preamplification, the lncRNA-generated Cq values ranged from 18.7 to 26.5 (mean = 22.5), whereas in the non-preamplification workflow, the results ranged from a Cq of 27.7 to 35.5 (mean = 32.6). The difference in the mean Cq between the workflows was 10.1.

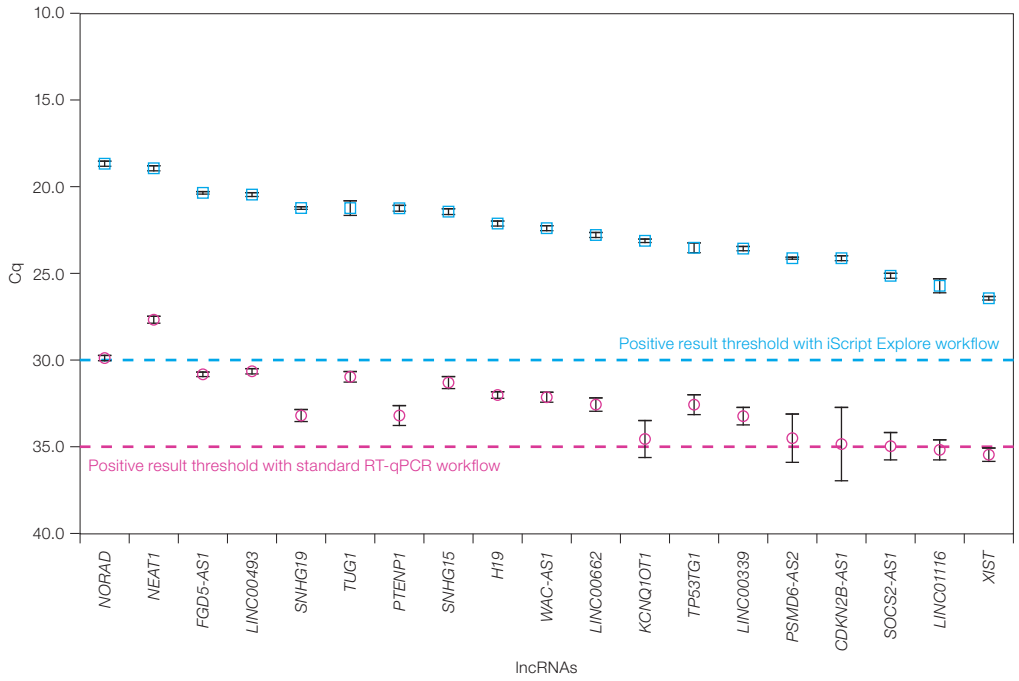


**Fig. 3. Improved detection of lncRNA using the iScript Explore One-Step RT and PreAmp Kit.** Universal human reference RNA (0.095 ng/µl; Agilent) was analyzed with preamplification (□) and non-preamplification (○) workflows. The input sample volume was maximized for both methods. For the preamplification method, 1 ng of reference RNA was preamplified for 14 cycles in a 50 µl reaction and was subsequently diluted tenfold to 500 µl. For the non-preamplification method, cDNA was generated in a 20 µl iScript gDNA Clear reaction from 1.33 ng reference RNA. One microliter of the resulting cDNA from each method was used as input for 10 µl qPCR reactions. The mean Cq for the 19 targets was 22.6 with the method using preamplification while the mean was 32.7 for the method without preamplification. The mean difference in Cq between the methods was 10.1. Error bars represent the standard deviation of the Cq from four technical replicates. Cq, quantification cycle.

## Discussion

Simultaneous detection of multiple targets expressed at low levels can present a serious challenge to researchers. By combining the unparalleled sensitivity of qPCR, with its wide linear dynamic range, with preamplification, researchers are able to boost the concentration of their targets. This allows sensitive quantification even in very limited samples.

Using the iScript Explore One-Step RT and PreAmp Kit in conjunction with PrimePCR Assays, we generated preamplified cDNA directly from 1,600 pg of cfRNA derived from human plasma samples collected from patients with prostate cancer and from healthy donors. With the preamplification protocol that was used, each copy of RNA added to the iScript Explore reaction is expected to in turn deliver approximately 33 copies of resulting cDNA (assuming optimal efficiency) to each downstream qPCR reaction. This means that each qPCR reaction effectively interrogated the entire 1,600 pg of input sample used in the preamplification reaction, which enabled the detection of an average 20 of the 41 lncRNA targets (49%) and 6 of the 15 mRNA targets (40%). To put those results in context, we can look at the comparison of the iScript Explore Kit–based workflow with the standard RT-qPCR workflow, where we saw that on average an lncRNA was detected 10.1 cycles earlier using the preamplification workflow. This shift is partly due to a 14.9-fold net increase in the amount of sample analyzed with the iScript Explore workflow and partly due to the additional copies of each target present in the qPCR for each target added to the iScript Explore reaction (~33:1). Taken together, the workflow using iScript Explore is expected to have 491-fold more of each target added to the qPCR, which would be expected to yield Cq values 8.9 cycles earlier. The average difference of 10.1 cycles may suggest that the gene-specific reverse transcription in the iScript Explore Kit is also more efficient than the random and oligo(dT)-primed reverse transcription in the iScript gDNA Clear Kit. By extrapolating the results of the method comparison to the plasma RNA sample analysis,

we can see that many of the targets analyzed in the cfRNA from plasma would have been considered negative in the standard workflow. Of the 29 lncRNA targets detected in at least one sample, 23 (79%) would have been expected to yield, on average, results beyond cycle 35 in the standard workflow. Similarly, for the eight mRNA targets detected in the cfRNA using the preamplification workflow, six (75%) would be expected to generate results beyond cycle 35 on average without preamplification.

In the analysis of HeLa and K562 cell lines, 107 lncRNAs were analyzed directly from cell lysates prepared using the SingleShot Cell Lysis Kit. Cell lysate from approximately 500 cells was used as the input for the iScript Explore One-Step RT and PreAmp reaction, and was in turn analyzed by each downstream qPCR reaction. From this amount of cell lysate we found that we were able to detect most of the lncRNA targets that were analyzed: 96 (90%) in HeLa cells and 94 (88%) in K562 cells. While cultures of immortalized cell lines are abundant and do not typically require the use of preamplification, analyzing cell culture lysates directly by RT-qPCR means that there is no purification step to concentrate the RNA. The use of this iScript Explore Kit–based preamplification workflow allowed us to detect approximately nine out of ten lncRNA targets in both cell lines.

## Conclusions

Using the iScript Explore One-Step RT and PreAmp Kit to generate preamplified cDNA in a single step provides benefits that are twofold for qPCR analysis of low-abundance targets: a given amount of sample can be analyzed for more targets, and there is an increase in the net amount of sample that can effectively be analyzed. Taken together, these benefits provide researchers with a means to analyze many low-abundance targets, such as lncRNA, in limited or dilute sample types, thus paving the way for the discovery of new RNA biomarkers. This is of particular interest in the search for pathology-associated markers, especially in the oncology field where detecting the aberrant expression of tissue-specific lncRNAs may lead to new RNA markers for liquid biopsies.

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